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THE CLOSER MUSCLE IN THE DIMORPHIC CLAWS OF MALE FIDDLER CRABS

C. K. GOVIND[1], M. M. QUIGLEY[2], AND K. M. MEAROW[1]

[1]*Department of Zoology, Scarborough Campus, University of Toronto, 1265 Military Trail, Scarborough, Ontario M1C 1A4, Canada, and* [2]*Department of Biology, University of Virginia, Charlottesville, Virginia 22901*

Abstract

The paired claws in male fiddler crabs are asymmetric consisting of a major and a minor type. The homologous closer muscle in these claws are innervated by two excitatory axons which, because they elicit tonic closing of the dactyl with repetitive stimulation and have neuromuscular synapses which facilitate, are regarded as slow type axons. The fiber composition of the paired closer muscles is also similar, being of the slow type. These fibers show typically long sarcomere (10 μm) and A-band (6 μm) lengths, light staining for myofibrillar ATPase denoting low specific activity for this enzyme, but more intense and circumscribed staining for NADH-diaphorase denoting a robust oxidative capacity. However, the protein composition of the paired muscles varies not only in its myosin light chain and paramyosin but also in the expression of isoforms for myosin heavy chain and several regulatory proteins including troponin I, tropinin T, and tropomyosin. Apart from any subtle functional differences imparted by these protein isoforms, the paired closer muscles are homogenously slow in their innervation and fiber type composition.

Introduction

In the past two decades a number of studies describing the neuromuscular organization in the dimorphic claws of crustaceans have emerged (see reviews by Mellon, 1981; Govind, 1984); studies prompted largely by our intrinsic interest in the asymmetry of body parts in an otherwise bilaterally symmetric animal. These studies have explored the cellular nature of the asymmetry in the paired closer muscles. In lobsters and snapping shrimps, these muscles are composed of slow fibers in the major claw and a mixture of fast and slow fibers in the minor claw. Curiously, in the lobster *Homarus americanus,* the paired muscles and their claws are symmetrical on hatching and only during subsequent juvenile development become asymmetric in a random manner *i.e.,* the major cheliped can occur either on the right or left side (Emmel, 1908; Lang *et al.,* 1978). The paired claws in very young, immature fiddler crabs are symmetrical and remain so as paired minor claws in females but become differentiated into a major and a minor claw in males (Morgan, 1924; Yamaguchi, 1977). The fiddler crabs therefore provide another example, apart from the lobster, where the acquisition of asymmetry in homologous structures may be studied.

However, there is little information on the paired asymmetric claws of male fiddler crabs apart from the above-mentioned work on the acquisition of asymmetry and

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Abbreviations: ATP, Adenosine triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol, bis (β-aminoethyl ether); N,N,N, N-tetra-acetic acid; NADH, nicotineamide dinucleotide, PMSF, phenyl methylsulfanyl fluoride; SDS, sodium dodecyl sulfate.

their behavioral role in feeding, aggression, and courtship (Crane, 1975). Huxley (1932) reported the unpublished observation of Ratcliffe that the segmental ganglia and nerve roots of the major claw were twice the size of those of the minor claw. More recently hypertrophy of the somata of the motoneurons and hyperplasia of sensory axons to the major claw have been documented (Young and Govind, 1983). Finally, Rhodes (1977) compared the fiber composition of several homologous muscles from the thorax and proximal segments of the claws in several species of fiddler crabs. He did not examine the claw closer muscle. Since this is the largest muscle in the claw and housed in the largest and most elaborate segment of the cheliped, it has been an obvious target for the study of asymmetry in other crustaceans such as snapping shrimps (Mellon, 1981), hermit crabs (Stephens *et al.,* 1984), blue crabs (Govind and Blundon, 1985), and lobsters (Govind, 1984). Therefore from a comparative viewpoint it seemed desirable to study the paired closer muscles in male fiddler crabs particularly since these animals provide the most flamboyant example of claw asymmetry (with the major claw being up to 50 times larger than its minor counterpart) amongst crustaceans studied so far. In addition, the correlation of contractile protein composition and muscle fiber type has recently been investigated in a number of crustaceans (Costello and Govind, 1984, Quigley and Mellon, 1984, Mykles, 1985a, b). The present study reports on the excitatory innervation, fiber type composition, and muscle protein expression patterns of the claw closer muscle in the male fiddler crab, *Uca pugnax.*

Materials and Methods

Adult male fiddler crabs (*Uca pugnax*) were collected during the summer from salt marshes around Falmouth, Massachussetts, and held in the laboratory in natural or artificial seawater at 20–24°C. They were fed frozen brine shrimp and fish *ad libitum.*

The motor innervation of the claw closer muscle was determined using electrophysiology for which the cheliped was removed from the animal and dissected in marine animal saline (Govind and Lang, 1981). The leg nerve was exposed in the carpus and stimulated with a pair of fine platinum wire electrodes or a suction electrode. The resulting activity in the closer muscle was recorded as myograms with a pair of copper wires inserted into the muscle through the exoskeleton and insulated except at the tips. Simultaneously, intracellular recordings from single muscle fibers were made using conventional glass microelectrodes. For this purpose small windows were cut in the propus to expose selected portions of the closer muscle. The experiments were made at 15–16°C.

The fiber composition of the closer muscle was determined in two different ways. The first was to measure the sarcomere and A-band lengths of muscle fibers. The closer muscle was exposed by removing the overlying opener muscle and exoskeleton and fixed in alcoholic Bouins fluid for 24 hours. During this period the dactyl was held in a half-opened position to simulate a resting state of the closer muscle. Following fixation, the closer muscle was cut into quarters and stored in 70% alcohol. Measurements of the average sarcomere length (SL) and A-band lengths in these fixed myofibrils were made according to techniques conventional to our laboratory (Govind *et al.,* 1977).

The second way to type fibers was through a histochemial demonstration of myofibrillar adenosinetriphosphatase (ATPase) activity. To do this, the thickened exoskeleton of the propus of the major claw was ground down to almost the hypodermis with a high speed hobby drill. The minor cheliped did not warrant such pre-treatment. Next, the entire claw was frozen, cut in 16 μm cross-sections, and stained for myofi-

brillar ATPase activity by techniques described elsewhere (Ogonowski *et al.*, 1980). In addition the oxidative capacity of muscle fibers was assessed by staining some sections for NADH diaphorase activity. This latter technique reveals the density of mitochondria which is the principal site of the oxidative capacity of the muscle fibers.

Protein composition of the two cheliped muscles was also compared using one-dimensional SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Closer muscles were dissected, frozen in liquid nitrogen, and shipped on dry ice to Charlottesville, Virginia, from the Marine Biological Laboratory in Woods Hole, Massachusetts. All protein purification procedures were done on ice. Whole muscle tissue samples were prepared by homogenizing frozen muscle (one major or 5–10 minor closer muscles) in 40 volumes of solution A (40 m*M* NaCl, 5 m*M* phosphate buffer, pH 7.2; 1.0 m*M* MgCl, 0.1 m*M* EGTA, 0.1 m*M* DTT, 0.5 m*M* PMSF, 0.18/ml leupeptin, 1.0/ml α-macroglobulin). Immediately after homogenization an equal volume of 2× gel sample buffer was added and the samples were boiled. Actomyosin was made by centrifuging muscle homogenates, washing with solution A two additional times, and resuspending the washed myofibrils in solution B (0.6 *M* KCl, 0.01 m*M* MgCl, 5 m*M* phosphate buffer, pH 7.2, 0.1 m*M* EGTA, 0.1 m*M* DTT) for 30 minutes. The high salt insoluble fraction was removed by centrifugation and the actomyosin precipitated by dialysis against solution A. Pelleted actomyosin was resuspended at 100 mg wet muscle weight/1 ml of gel sample buffer. Myosin samples were generated essentially as described by Chantler and Szent-Györgyi (1980). Final myosin pellets were resuspended in a sample buffer at 1 mg/ml.

Whole muscle, actomyosin, and myosin samples from major and minor claws were compared on 12.5% acrylamide gels or 4–5% gradient gels and visualized with Coomassie blue stain.

Results

Excitatory innervation

Innervation to the closer muscle was determined by stimulating the leg nerve in the carpus and monitoring the resulting myogram. Two distinct electrical responses with different latencies were recorded (Fig. 1A) suggesting the presence of two excitor axons to the closer muscle. We refer to these as E1 and E2 axons with E1 giving rise to the shorter latency myogram than E2. Furthermore, intracellular recordings in single fibers confirmed the presence of two distinct excitatory junctional potentials (ejps) corresponding to the extracellulary recorded myogram (Fig. 1B). Thus excitatory innervation to the closer muscle in fiddler crabs conforms to the established brachyuran pattern of two axons (Wiersma, 1961). No attempt was made to determine the inhibitory innervation to the closer muscle.

Stimulation of each axon elicited no visible twitch contractions with single or twin pulses but produced slow (tonic) contractions with repetitive stimulation at 5 Hz and higher. On this basis alone, both axons can resemble the slow or tonic type axons characteristic of the slow closer muscle in other brachyurans such as crayfish and lobsters, where the two excitors are differentiated into a fast and slow type.

The ejps evoked by each of the two axons were examined principally among the dorsal fibers and a few, more deeply located fibers. The axons responded in a similar fashion in both major and minor chelipeds. Usually E1 evoked a relatively small ejp seen only with repetitive stimulation when it would attain an amplitude of 1–2 mV, (Fig. $1B_1$, C). The E2 axon, on the other hand, gave with a single pulse an ejp of approximately 2 mV. With twin pulse stimulation (6–8 ms interpulse interval) this

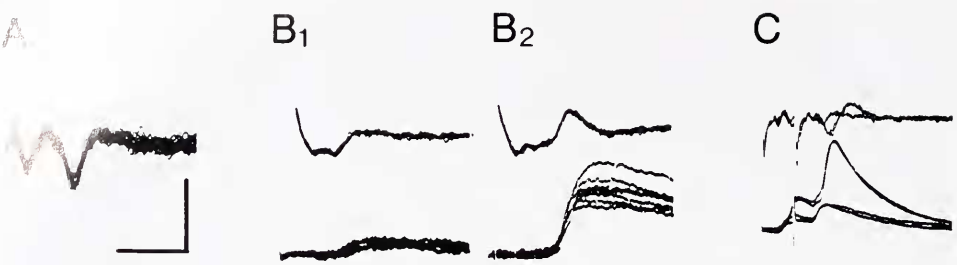

FIGURE 1. Excitatory innervation of closer muscle in minor claw. A; two distinct myograms in closer muscle with stimulation of cheliped nerve denoting innervation by two axons. $B_{1,2}$; myograms (upper traces) with different response times and their corresponding ejps (lower traces) in a single muscle fiber. Note the several sweeps in each ejp record showing little facilitation in B_1 but considerable facilitation in B_2. C; composite trace showing ejps (lower traces) and myograms (upper traces) to each of two axons stimulated separately and with twin pulses (separated by 6 ms) to show facilitation of ejps. Calibration, horizontal. A, B 8 ms, C 20 ms; vertical B, C 4mV.

ejp facilitated 3–5 fold in both claws (Fig. $1B_2$, C). In our limited sampling of fibers there did not appear to be any difference in the degree of synaptic facilitation between major and minor chelipeds. Synapses from both axons did not show any signs of fatigue when stimulated at 5 or 10 Hz for several minutes.

Fiber type composition of muscle

A well-established method for typing crustacean fast and slow fibers is by their characteristic sarcomere length (SL), with relatively short lengths (<4 μm) being indicative of fast-contracting fibers and longer lengths (>6 μm) of slow contracting fibers (Atwood, 1976). On this basis the closer muscles of the major and minor claw are composed of slow fibers having sarcomere lengths greater than 6 μm (Fig. 2). The muscle was divided into four areas and 30–40 measurements were made from each area revealing no differences among the areas. For the major claw muscle 159 measurements were made yielding a SL of 9.68 $\pm$ 1.09 and an A-band length of 6.15 $\pm$ 0.74 (X $\pm$ S.D.). For the minor claw muscle 100 measurements were made yielding a SL of 9.58 $\pm$ 1.45 and an A-band length of 6.12 $\pm$ 0.92. Since the A-band is less subject to variations in length than the sarcomere, the present results suggest a single population of long-sarcomere slow fibers in the closer muscles of both major and minor chelipeds. There are, however, several peaks in the distribution of A-band and sarcomere lengths in both muscles but whether these represent sub-types within the broad category of slow fibers is unknown. However, differentiation into sub-types also is suggested by histochemical evidence (see below). The distribution pattern in SL shown in Figure 2 was confirmed in three other animals where fewer measurements were taken.

The staining intensity for myofibrillar ATPase was uniform over the entire closer muscle as revealed in serial cross-sections of both minor and major claws (Fig. 3, upper row), in all six animals examined. This, combined with the fact that the fibers have long sarcomere lengths, strongly suggests that the closer muscle is of the slow variety. Further confirmation for this conclusion was obtained when myofibrillar ATPase activity was determined simultaneously in the closer muscles in the claws of fiddler crabs and in the walking legs of lobster, *Homarus americanus*. The closer muscle in the lobster has distinct fast and slow fiber populations (Govind *et al.*, 1981)

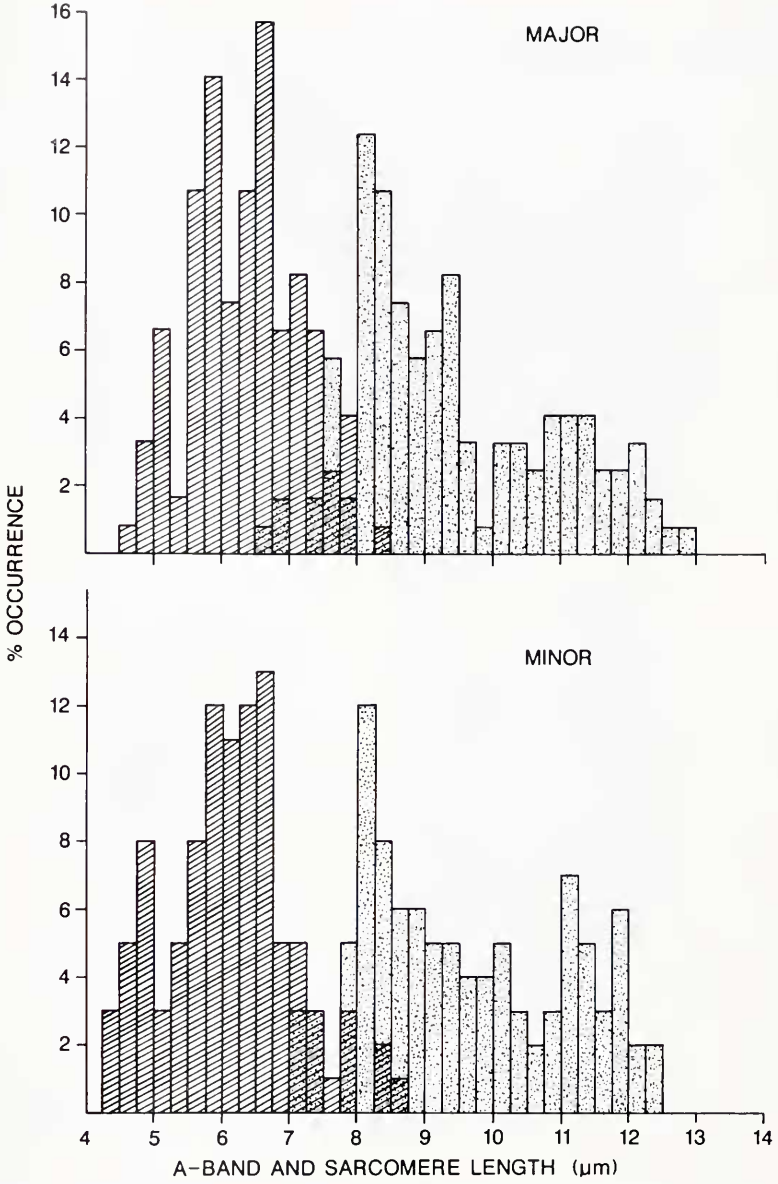

FIGURE 2. Percent distribution of closer muscle fibers with characteristic A-band (hatched) and sarcomere (stippled) lengths from a major and minor claw. Note the close similarity in distribution of these parameters between the two chelipeds. Number of measurements of A-band and sarcomere lengths is 159 and 100 for the major and minor claws respectively.

and we were able to judge that the staining intensity of the fiddler crab fibers resembled those of the lobster slow type. Thus, based on ATPase activity, the claw closer muscle in fiddler crabs is of the slow variety.

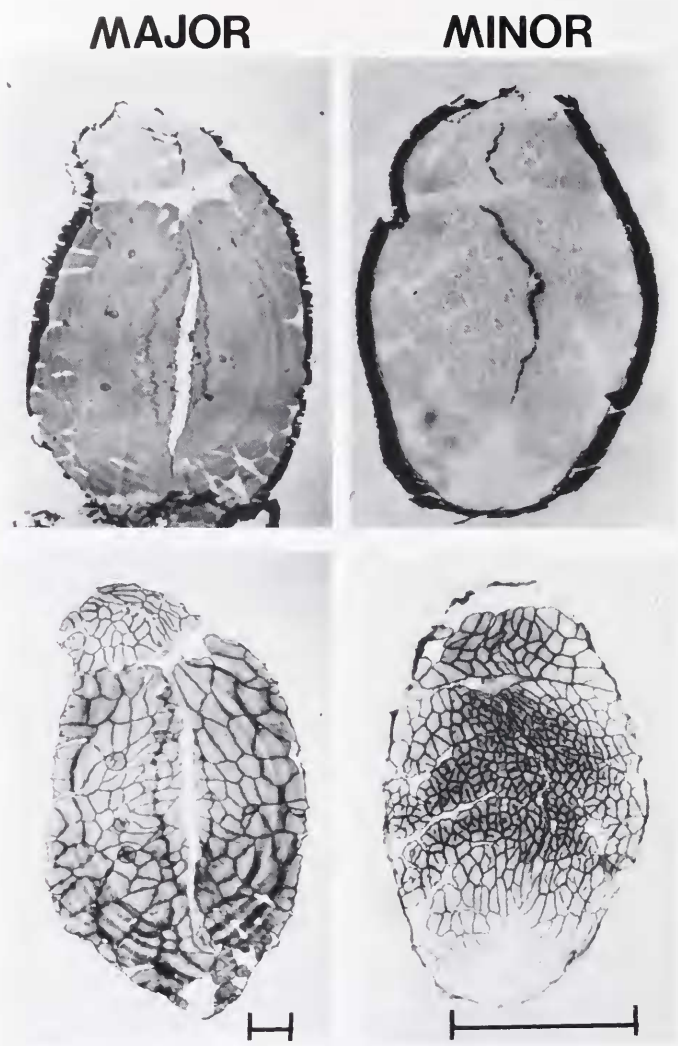

FIGURE 3. Representative cross-sections stained for myofibrillar ATPase (upper row) and NADH-diaphorase (lower row) from the mid region of the major and minor claws. The opener muscle is dorsally situated and smaller compared to the closer muscle which occupied most of the claw. Magnification 25×. Scale bar = 1mm.

However, subtle variation in staining intensity suggests differentiation of the slow fibers into sub-types. In the major claw muscle alone, a few distal fibers on either side of the tendon stain less intensely than the majority, suggesting a slower variety. But the most striking variation in the major claw is between the closer and opener muscle, with the latter staining much less intensely than the former (Fig. 3, upper row). The opener fibers appear to be of a much slower variety than the closer fibers. The ATPase staining intensity is similar between the opener and closer muscles in the minor claw. The opener muscle shows a regional distribution in staining intensity with the most

lateral fibers staining slightly darker than the central ones. These subtle variations in ATPase activity suggest sub-types within the slow fiber category as has been previously shown in lobster muscle with histochemical (Kent and Govind, 1981) and biochemical (Mykles, 1985b) techniques.

To examine the oxidative capacity of the closer muscle fibers, frozen sections were stained for NADH diaphorase (Fig. 3, lower row) which is located principally in the mitochondria. These organelles tend to be most abundantly distributed around the periphery of muscle fibers and staining was restricted to this area in the major claw muscles. However some distal fibers in the major closer muscle revealed a higher density of mitochondria and as these were the same fibers which stained less intensely for ATPase, they represent a slower variety than the remainder. Similarly in the opener muscle of the minor claw, a much slower variety was recognized by the more densely stained profiles of fibers around the tendon compared to their counterparts at the lateral edges (Fig. 3, lower row). The closer muscle in the minor claw also revealed regional differentiation in oxidative capacity: fibers around the tendon showing a much higher density of mitochondria than those located more peripherally (Fig. 3, lower row). Clearly there is variation in both oxidative capacity and ATPase activity among the slow fibers of the closer and opener muscle suggesting further differentiation into sub-types within the slow category.

Identification of muscle protein isoforms

Until recently no identification of contractile protein isoforms had been reported in crustacean muscle. A number of investigators have now identified isoforms of the major contractile proteins by applying standard muscle protein purification procedures (Costello and Govind, 1984; Quigley and Mellon, 1984; Mykles, 1985a, b). We have taken this approach to compare contractile protein expression in the major and minor claw closer muscles to other crustacean muscles of known fiber type.

Figure 4 shows whole muscle, actomyosin, and myosin samples separated by SDS-polyacrylamide gel electrophoresis. Myosin light chain components have been identified by their presence in purified myosin samples. Both cheliped muscles contain a 15 K light chain (LC1) and 23 K doublet (LC2). We detect no difference in mobility of these components. This pattern is similar to that observed by Costello and Govind (1984) in lobster claw closer muscles. There is, however, an additional 21 K protein present in the minor claw myosin sample that is absent in the major claw (Fig. 4f). This protein can be detected in whole muscle samples prepared by homogenization in buffer containing protease inhibitors, followed by immediate boiling. Therefore this band is thought not to be a breakdown product and represents an intact component of the minor claw closer muscle.

A rather large number of putative troponin I (TnI) isoforms have been identified in crustacean muscles. Mykles (1985a) has resolved five TnI variants in different combinations in lobster muscles, and three TnI isoforms have been tentatively identified in *Alpheus*. In the region where TnI is expected to migrate we observed two bands in *Uca* actomyosin preparations. Both forms are present in each muscle (Fig. 4a–d), but in the major claw the faster-migrating, 25 K band predominates, whereas in the minor muscle the 26 K isotype is the major form. A similar distribution of TnI variants was reported in the claw closer muscles of *Homarus* (Costello and Govind, 1984). In that case it was concluded that these were fiber type specific isoforms.

Troponin T (TnT) and tropomyosin (Tm) subunits also exhibit heterogeneity in the cheliped muscles of *Uca*. Crustacean TnT and Tm migrate with apparent molecular

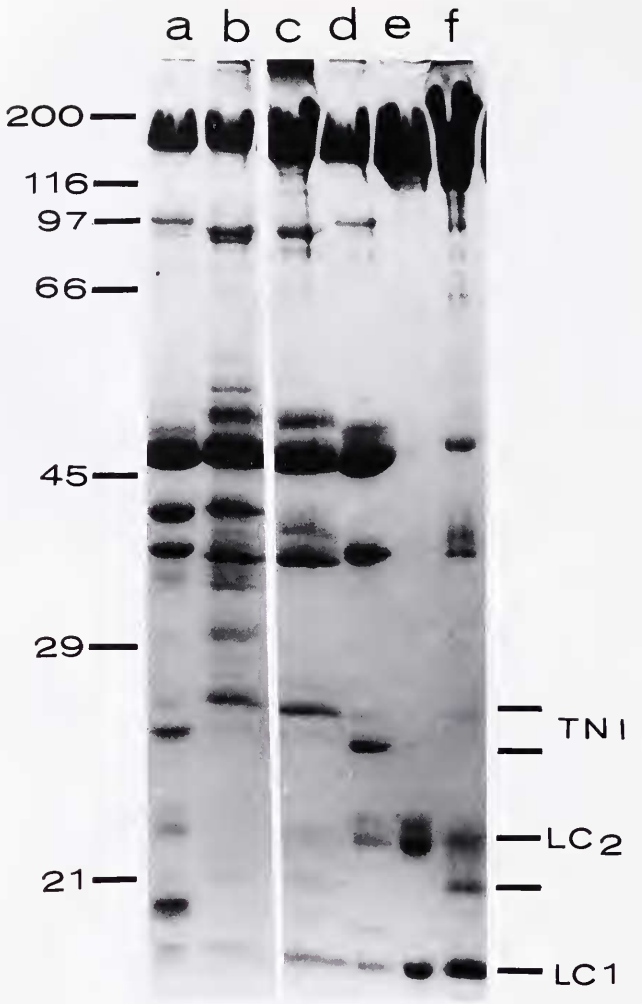

FIGURE 4. Whole muscle samples of major (a) and minor (b), actomyosin samples of minor (c) and major (d), and myosin samples of major (e) and minor (f) claw closer muscles, separated on a 12.5% acrylamide gel. Relative mobilities of markers of known molecular weight (×1000) are indicated on the left while major proteins of myosin light chain 1,2 (LC1,2) and troponin I (TN1) are indentified on the right.

weights near 52 K and 38 K respectively (Regenstein and Szent-Györgyi, 1975). In *Alpheus,* TnT expression in the asymmetric claws has been shown to be specific to claw type and not to fiber type *i.e.,* the pincer closer muscle which is made up of fast and slow fibers has a single TnT isotype, while the snapper muscle composed of all slow fibers, has another distinct form (Quigley and Mellon, 1984). The same pattern is observed in *Uca.* In Figure 5 *Alpheus* snapper and pincer closer muscle actomyosin samples were electrophoresed next to *Uca* closer actomyosin samples. With respect to TnT, the fiddler crab major claw appears similar to the pincer of *Alpheus,* since it contains a faster migrating subunit. The fiddler crab minor muscle TnT migrates more

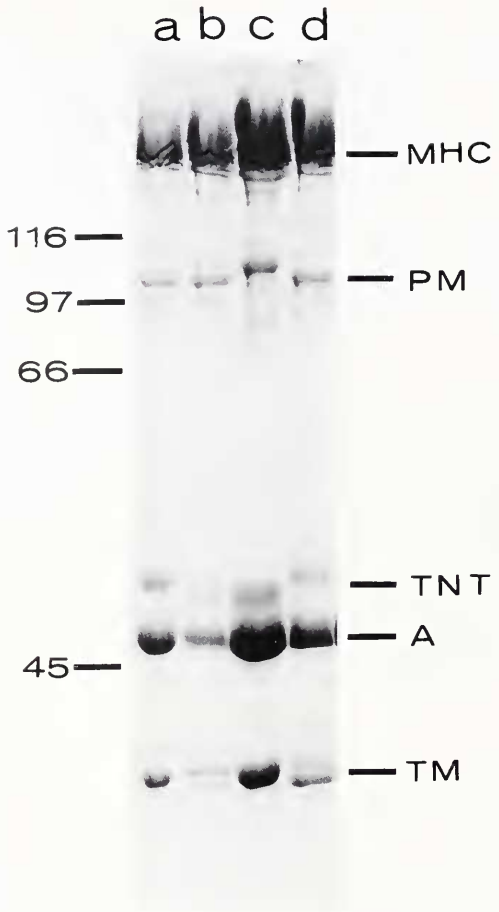

FIGURE 5. Actomyosin samples from the snapper (a) and pincer (b) cheliped closer muscles of *Alpheus* compared to those from the major (c) and minor (d) muscles of *Uca*. Samples were run on a 12.5% acrylamide gel for an extended period (8 h) to increase resolution in the 30–60 K range. Major proteins identified tentatively include myosin heavy chain (MHC), paramyosin (PM), troponin T (TNT), actin (A), and tropomyosin (TM).

slowly, similar to the snapper form in *Alpheus*. Interestingly the similarities are reversed for Tm expression. The fiddler crab minor closer muscle has a doublet at the Tm position, with each isoform present in apparently equimolar amounts. This is the same pattern observed in the snapping shrimp pincer claw. In the major claw of the fiddler crab and in the snapper claw of *Alpheus*, the larger subunit is found in excess (Fig. 5).

In *Homarus* (Mykles, 1985a) and *Alpheus* (Fig. 5) the expression of paramyosin isoforms has been correlated with muscle fiber type. We report differences in the protein banding patterns in claw muscles of *Uca* in regions where paramyosin is expected to migrate (Fig. 5). The minor muscle contains a faster-migrating component than the major muscle. Therefore it appears that crustacean slow muscles are not required to express the faster-migrating paramyosin isoforms.

The large size of the myosin heavy chain (MHC) has made detecting differences

FIGURE 6. Separation of increasing amounts of myosin heavy chain (MHC) isoforms from minor (a, c, e) and major (b, d, f) cheliped muscles in low percentage acrylamide gels. Four to five percent gradient gels were run with whole muscle samples. Only that region of the gels where MHC runs is shown since most major components had run off the gel. Loading series consisted of approximately 0.5, 1.0, and 2.0 g of MHC for each muscle.

with standard one-dimensional gel electrophoresis difficult. Recently, however, low percentage acrylamide gels have been used to resolve MHC isoforms (Carraro and Catani, 1983). We have found that 4–5% gradient gels yield the best resolution. Figure 6 shows the MHC region of such a gel. A single MHC isoform is detected in the small claw, while two bands are present in the large claw sample. The samples loaded are whole muscle preparations so that differences are very likely not due to modifications introduced during purification. This represents the first demonstration of MHC isoforms in crustacean muscles. It is unclear what role this difference in MHC composition plays since no major differences in sarcomere length of myofibrillar ATPase activity have been observed in the claw muscles.

DISCUSSION

All of the properties of the closer muscle in the paired claws of the male fiddler crab explored in the present study are of representative slow type fiber. This is best exemplified by the muscle in which the fibers typically have long SL, low ATPase activity, and high oxidative capacity. Moreover, their protein composition resembles those of other crustacean slow muscle. Thus apart from differences in the isoforms of some contractile and regulatory control proteins, for which the functional significance is unknown, the closer muscle in the major claw is identical to its homolog in the minor claw.

Excitatory innervation of the closer muscle is similar on the two sides. Both muscles are innervated by slow axons which give rise to neuromusclar synapses which facilitate markedly and bring about tonic closing of the claw with repeated firing.

The similarities in neuromuscular properties between the paired muscles are somewhat surprising in view of striking asymmetry in the size and behavior of the claws. On the question of size, the major claw is some 30–50 times larger than the minor (Rhodes, 1977). Associated with this enlarged size of the major claw is a corresponding hypertrophy of the closer muscle, motoneuron somata, and hemiganglion, and a hyperplasia of sensory axons (Young and Govind, 1983). On the question of behavior, it is well-known that the minor claw is used for feeding while the major one is for courtship and defense (Crane, 1975). During feeding the closing action of the dactyl would be employed for gathering the substrate while other more basal segments of the claw would move the claw to the mouth where the substrate is deposited. These small basal segments would bring about the characteristic waving motion of the major claw during courtship. The closing action of the major claw would be used more in

defense *e.g.*, arresting an intruder. Closing of the dactyl does not have to be unusually rapid in either of these feeding or defense situations. Consequently it is not surprising to find a slow type neuromuscular system in both the major and minor claws.

The degree to which homologous neuromuscular systems diverge in the dimorphic claws of crustaceans varies considerably. The present study of the paired closer muscles in male fiddler crabs shows little if any divergence except for some protein isoforms. In comparison the neuromuscular systems in the dimorphic claws of blue crabs *Callinectes sapidus*, are slightly more divergent (Govind and Blundon, 1985). Although the fiber composition of the paired closer muscles is of the slow type, the motor axons to the minor claw are effective at a lower frequency of firing than their homologs in the major claw. Moreover the excitatory axons to the closer muscles are differentiated into fast and slow types unlike those in the fiddler crab where both axons are of the slow type. Divergence of not only the motor axons but of the closer muscles is seen in hermit crabs (Stephens *et al.*, 1984), snapping shrimps (Stephens and Mellon, 1979; Stephens *et al.*, 1983), and lobsters (Lang *et al.*, 1977; Ogonowski *et al.*, 1980). In all of these species, the major closer muscle is composed entirely of slow fibers while the minor muscles has a mixture of fast and slow fibers. The percentage of fast fibers in the minor closer muscle is approximately 20–40% in snapping shrimps (Govind *et al.*, 1986), 40–50% in hermit crabs (calculated from data on sarcomere lengths provided by Stephens *et al.*, 1984), and 60–80% in lobsters (Govind, 1984). These figures show a tendency toward differentiating a largely fast neuromuscular system in the closer muscle of the minor claw in contrast to the major claw where it is entirely slow. Consequently the paired homologous neuromuscular systems of the claw closer muscles diverge from a symmetric state in blue crabs to an almost completely asymmetric state in lobsters.

Finally, the identification of protein isoforms in the closer muscles of fiddler crabs warrants some comment. Vertebrate muscle has been shown to contain fast and slow fiber type specific isoforms for most of the major contractile proteins (Jolesz and Sreter, 1981). Costello and Govind (1984) reported a correlation of isoform expression and fiber type in the claw closer muscles of the lobster. However these were the only two muscles examined. Mykles (1985a) subsequently reported, in a study of contractile protein expression in six lobster muscles, that protein composition of a muscle is not simply correlated with fiber types. This observation was also made when isotypes in homogenous slow, homogenous fast, and mixed muscles of *Alpheus* were compared (Quigley and Mellon, 1984). We have extended these findings to show that the homogenous slow claw closer muscles (as judged by morphology, physiology, and histochemistry) of *Uca* are heterogenous for a number of contractile protein isoforms. These include TnT, TnI, Tm, and MHC.

The meaning of these contractile protein isoform differences, in the face of no observable differences in contractile properties, ATPase staining, or sarcomere length, raises the question of what function the various isoforms play. One possibility is that the observations of physiological, histochemical, and morphological properties fail to identify subtle but significant differences in muscle function served by these proteins. Alternatively, some examples of fiber type or muscle-specific protein expression may be the consequence of the organization of muscle genes into coordinately regulated sets (Davidson and Britten, 1973) which may ensure the proper development and assembly of a muscle. It is possible that the various patterns of contractile protein isoform expression seen in the asymmetric claw closer muscles of crustaceans results from the grouping of contractile proteins into sets of large or small claw specific components. Clearly a better understanding of crustacean muscle protein biochemistry

and the regulatory mechanisms for determining their patterns of expression is needed to address this question.

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